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Anti-microbial effect of *Saussurea costus* on multidrug-resistant bacteria

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ABSTRACT

Background and objective: Saussurea costus was widely used in traditional medicine to treat respiratory problems. Its anti-microbial effect was investigated in multiple studies. This work aims to examine the anti-microbial effect of ZnO-NPs of Saussurea *costus* on some respiratory bacteria. *Methods:* After preparation of methanolic extract of *Saussurea costus* roots, we synthesized zinc oxide nanoparticles (ZnO-NPs) using an aqueous extract from *Saussurea costus.* The anti-microbial effect against *Klebsiella pneumonia, Staphylococcus aureus, Acinetobacter baumannii, and Pseudomonas aeruginosa* and their reference strains were assessed through well plate agar diffusion method, minimal inhibitory concentration (MIC), and minimal bacterial concentration (MBC). *Results:* Both methanolic extract and Zn-ONPs exhibited anti-microbial activity with a variable diameter of inhibition zones, where the largest inhibition zone was observed against the standard strain *A. baumannii* ATCC 19606 followed by *A. baumannii* and *K. pneumonia* clinical isolates (20 ± 0.03, 16 ± 0.01, and 16 ± 0.00 mm, respectively). ZnO-NPs have MICs in the range of 3.75-7.5 mg/mL, while the MIC values of *S. costus* methanolic extract ranged from 3.75 mg/mL to 15 mg/mL. The MBC values were generally found to be twice the inhibitory concentrations. *Conclusions:* Saussurea *costus* methanolic extract and ZnO-NPs have an anti-microbial effect against multidrug-resistant bacteria.

Keywords: *Saussurea costus*, anti-bacterial effect, multidrug-resistant bacteria, nanoparticles

1. INTRODUCTION

Medicinal plants provide a generous source with a potent potential to treat multiple health problems (Rates, 2001). *Saussurea costus (S. costus)* is a plant that belongs to the Asteraceae family (El-Far et al., 2018). For centuries, costus was widely used in traditional medicine to treat respiratory problems (Elnawasany, 2022). Anti-microbial action of *S. costus* was evidenced against Shigella spp., *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas spp.*, B*acillus subtilis, and Salmonella spp.*, *Staphylococcus epidermidis*, *Enterobacter cloacae*,

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Enterococcus faecalis, and Acinetobacter baumannii (Malabadi, 2005; Ahmed and Coskun, 2023). The anti-microbial effect of zinc oxide nanoparticles (ZnO-NPs) was investigated in many studies.

Pomegranate extracts were used to synthesize ZnO-NPs. They were effective against multiple pathogenic strains such as *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* (Ifeanyichukwu et al., 2020). ZnO-NPs exerted a potent anti-microbial potential against multiple gram-negative and positive bacteria. This action is attributed to the downregulation of microbial growth and interfering with membrane permeability (Huh and Kwon, 2011). This work investigates the anti-microbial effect of methanolic extract of *S. costus* and ZnO-NPs on some respiratory bacteria.

2. METHODS

Preparation of plant material

S. costus roots were procured from the Al-Tameer local market in Riyadh, Saudi Arabia. After rinsing them with tap water, the roots were sectioned into bite-sized pieces and left to dry in a shaded area. Then, the roots were ground to extract using methanol. One hundred grams of the plant was soaked in 500 ml of solvent and placed on a VWR DS 500 orbital shaker for 72 hours (Vidya and Arulpandi, 2016; Ehsan and Sajjad, 2017). The produced extracts were filtered using Whatman No. 1 filter paper. Methanol was removed from the extracts through normal evaporation in a sterile laminar cabinet over 48 hours. Once completely dried, the extracts were stored at room temperature.

Synthesis of zinc oxide nanoparticles

S. costus roots aqueous extract and salt of zinc of Sigma-Aldrich Co. in Budapest, Hungary were used to formulate the ZnO-NPs. We prepared a mixture of twenty grams of grinned *S. costus* roots and 150 mL of distilled water that was boiled for a couple of hours at 80°C then filtered by Whatman No. 1 filter paper. After the addition of five grams of zinc nitrate and two hours of boiling, a brown-yellow-coloured product was obtained (Ehsan and Sajjad, 2017). We stored it at 60°C for one day and then we got a dark brown spongy paste. Finally, white powder of pure ZnO-NPs was formed after a two-hour heating at 400°C in a muffle furnace (Mohd et al., 2020).

Characterization

UV-vis characterization was done through UV-vis spectroscopy (UV-1800; Shimadzu UV Spectrophotometer, Kyoto, Japan). To produce ZnO-NPs with the required optical criteria and range at a resolution. Fourier-transform infrared spectroscopy (FTIR) of 400-4000 cm^{-1} range (PerkinElmer, Spectrum BX, Waltham, UK) was utilized to detect the vibrations and functional groups of *S. costus* aqueous extract and ZnO-NPs. Additionally, we tested the plant extract using both UV-VIS and FTIR to compare it with the synthesized nanoparticles. The surface morphology of nanoparticles was assessed using SEM (JSM-IT200; JEOL, Japan).

Assessment of the anti-microbial activity

We focused on four clinical isolates of multidrug-resistant organisms (MDROs) responsible for respiratory infections. These clinical isolates included *Klebsiella pneumoniae, Staphylococcus aureus, Acinetobacter baumannii, and Pseudomonas aeruginosa*. At the same time, we compared these clinical samples with reference strains obtained from the American Type Culture Collection (ATCC). The ATCC strains consisted of *Klebsiella pneumoniae* (ATCC 700603), *Staphylococcus aureus* (ATCC 29213), *Acinetobacter baumannii* (ATCC 19606), and *Pseudomonas aeruginosa* (ATCC 27853), Amoxicillin, meropenem, vancomycin, and cefixime were used as a control. Well plate agar diffusion method was applied to evaluate the anti-microbial action.

Following nocturnal incubation in nourishing broth, bacterial growth was adjusted to 0.5 McFarland turbidity standards. Subsequently, the examined bacteria were inserted on Mueller-Hinton agar (MHA) plates. Next, we inoculated approximately 1 mL of the tested bacteria and reference strains onto Mueller and Hinton agar. Then, six mm-diameter wells were made on the agar plates. A sonicated 7.5 mg/mL solution of 7.5 mg of ZnO-NPs and *S. costus* extract and 1 mL of Dimethyl sulfoxide (DMSO) was introduced to each well of bacteria-inoculated culture plates (Jayachandran et al., 2021). The zone of inhibition (ZOI) was determined after one day of incubation at 37°C. This experiment was repeated twice.

Moreover, we measured the minimum inhibitory concentration (MIC) using the broth macro dilution procedure according to the Clinical and Laboratory Standards Institute (CLSI) guidelines (Mohd et al., 2020). The minimum bactericidal concentration (MBC) was

determined by inoculating half mL from MIC tubes, which showed no observed bacterial growth signs onto sterile Mueller-Hinton agar. Finally, after incubating the MBC plates at 37°C for 24 hours, we determined the MBC by noting the concentration at which no visible growth was observed (Jain et al., 2011).

Statistical analysis

Data were analyzed using SPSS 20. Mean ± standard deviation was utilized to describe inhibitory zone diameters. The Kruskal-Wallis test was used to compare the diameters of the inhibitory zone. Mann Whitney test to compare MIC and MBC. P-value < 0.05 was considered significant and < 0.001 was considered highly significant.

3. RESULTS

Characterization of nanoparticles

UV-Visible Spectroscopy

The biosynthesis of ZnO-NPs from *S. costus* extract was initially confirmed by UV-visible spectroscopy. A distinct peak at 350 nm, a feature of ZnO-NPs, was visible in Figure 1, verifying their synthesis.

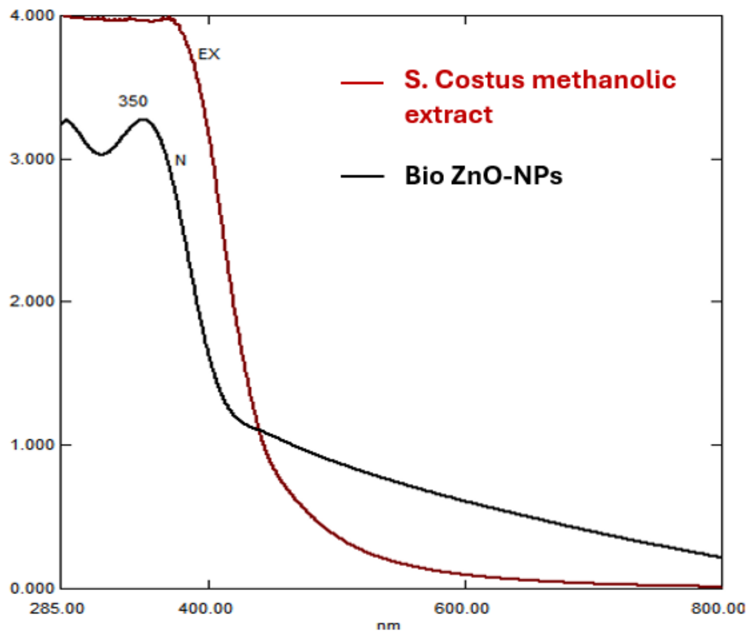

Figure 1 UV-visible absorption bands of S. costus ZnO-NPs

Fourier Transform Infrared (FT-IR) Analysis

The various functional groups involved in the stabilization and biosynthesis of the nanoparticles (NPs) were revealed by the FTIR spectrophotometer. The interaction of biological components of *S. costus* extract with nanoparticles indicated peaks at 3425.58, 2924.09, 1519.91, 1404.18, 1111.00, and 432.05 cm−1 (Figure 2). The existence of polyphenols was indicated by the broad spectrum at 3425.58, and the binding of ZnO with the OH group was suggested by the absorption peak found at 2924.09 cm−1.

The ethylene group is represented by the peaks at 1519.91 cm−1. The presence of protein amine II bands in the sample is responsible for the peak at 1404.18 cm−1. The detected band at 1111.00 cm−1, which is typically present in proteins and is engaged in the metal ion reduction process, could be the cause of the C-N stretching vibrations. Additionally, the distinctive ZnO peak, located at 432.05 cm−1, was visible in the FTIR absorption spectra, confirming that *S. costus* extract is responsible for the biogenesis of ZnO-NPs.

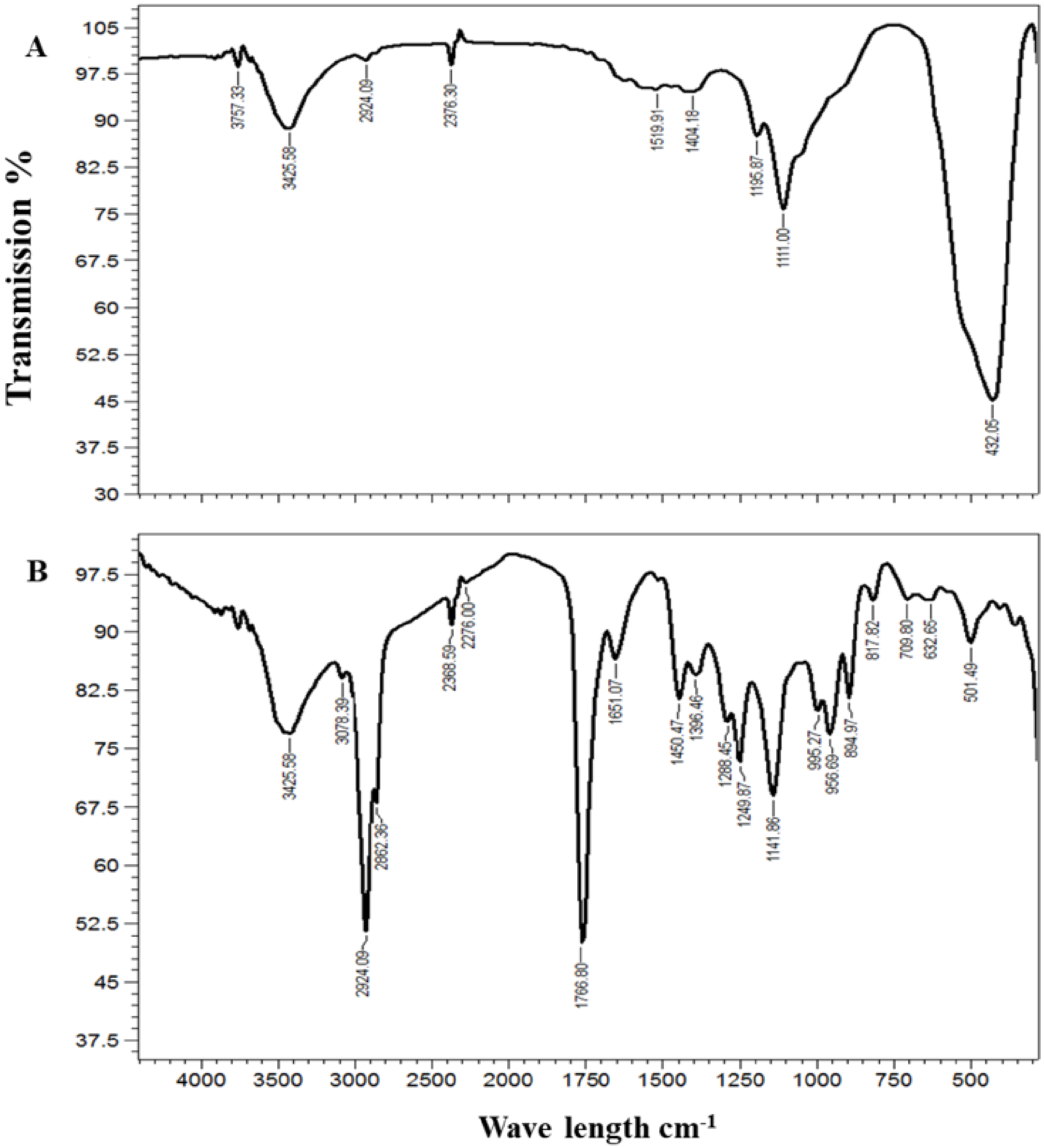

Figure 2 FTIR spectra of Bio-ZnO-NPs (A), and S. costus extract (B)

Scanning electron microscopy (SEM)

SEM images of the bio ZnO-NPs prepared by green synthesis using *S. costus* are represented in (Figure 3). Spherical nanoparticles with smooth surfaces, free from pores or holes, were observed. The size range measured from the SEM images was very narrow (19.5 - 37.5 nm). These results showed the homogeneity of the prepared NPs together with their very small nano size (Figure 3).

Anti-microbial activity of green synthesized ZnO-NPs

The anti-microbial effect of methanolic extract of *S. costus* and green synthesized ZnO-NPs with *S. costus* was investigated by agar well diffusion assay against selected bacterial strains (Table 1 & Figure 4). Both methanolic extract and ZnO-NPs exhibited anti-microbial activity with a variable diameter of inhibition zones, where the largest inhibition zone was obtained against the standard strain *A. baumannii* ATCC 19606 followed by *A. baumannii* and *K. pneumonia* clinical isolates (20 ± 0.03, 16 ± 0.01, and 16 ± 0.00 mm, respectively). *P. aeruginosa* was resistant to both ZnO-NPs and methanolic extract.

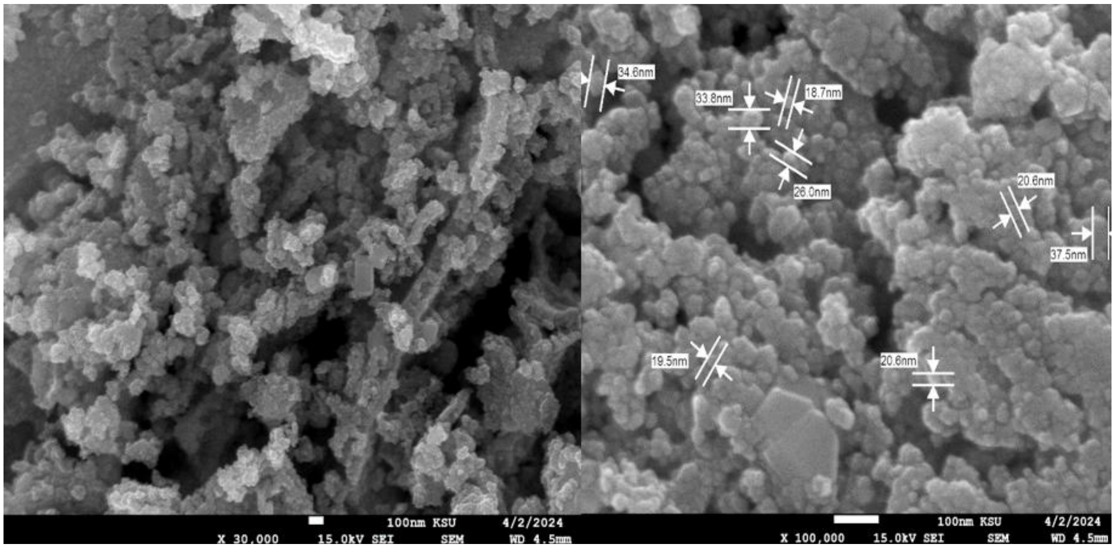

Figure 3 SEM images of ZnO-NPs

Table 1 Anti-microbial activity of ZnO-NPs of *S. costus* methanolic extract

Microorganisms	Inhibition zone diameter (mm)		*P*-value
	Methanolic extract	ZnO NPs	
K. pneumoniae	16 ± 0.01	16 ± 0.00	> 0.05
S. aureus	10 ± 0.04	0 ± 0.00	< 0.05
A. baumannii	16 ± 0.05	16 ± 0.01	> 0.05
P. aeruginosa	0 ± 0.00	0 ± 0.00	> 0.05
K. pneumoniae (ATCC 700603)	15 ± 0.01	11 ± 0.00	< 0.05
S. aureus (ATCC 29213)	13 ± 0.00	0 ± 0.00	< 0.05
A. baumannii (ATCC 19606)	11 ± 0.02	20 ± 0.03	< 0.05
P. aeruginosa (ATCC 27853)	8 ± 0.01	8 ± 0.01	> 0.05

However, *S. aureus* was resistant to ZnO-NPs. Methanolic extract achieved a higher inhibition zone against *A. baumannii*, then *K. pneumoniae* and *K. pneumoniae* (ATCC 700603) (16 ± 0.05, 16 ± 0.01, and 15 ± 0.01mm, respectively. Consequently, MICs and MBCs values were determined, as shown in (Table 2). We noticed that ZnO-NPs have MICs range of 3.75-7.5 mg/mL, while the MIC values of *S. costus* methanolic extract ranged from 3.75 mg/mL to 15 mg/mL. The MBC values were generally found to be twice the inhibitory concentrations (Table 1, 2), (Figure 4).

Table 2 MIC and MBC of ZnO -NPs of *S. costus* ethanolic extract

Microorganisms	MIC (mg/ml)		MBC (mg/ml)	
	Methanolic extract	ZnO NPs	Methanolic extract	ZnO NPs
K. pneumoniae	7.5	3.75	15	7.5
S. aureus	3.75	ND	7.5	ND
A. baumannii	3.75	7.5	7.5	15
P. aeruginosa	ND	ND	ND	ND
K. pneumoniae (ATCC 700603)	3.75	3.75	7.5	7.5
S. aureus (ATCC 29213)	7.5	ND	15	ND

A. baumannii (ATCC 19606)	3.5	3.75	7.5	7.5
P. aeruginosa (ATCC 27853)	15	7.5	30	15

ND: not detected.

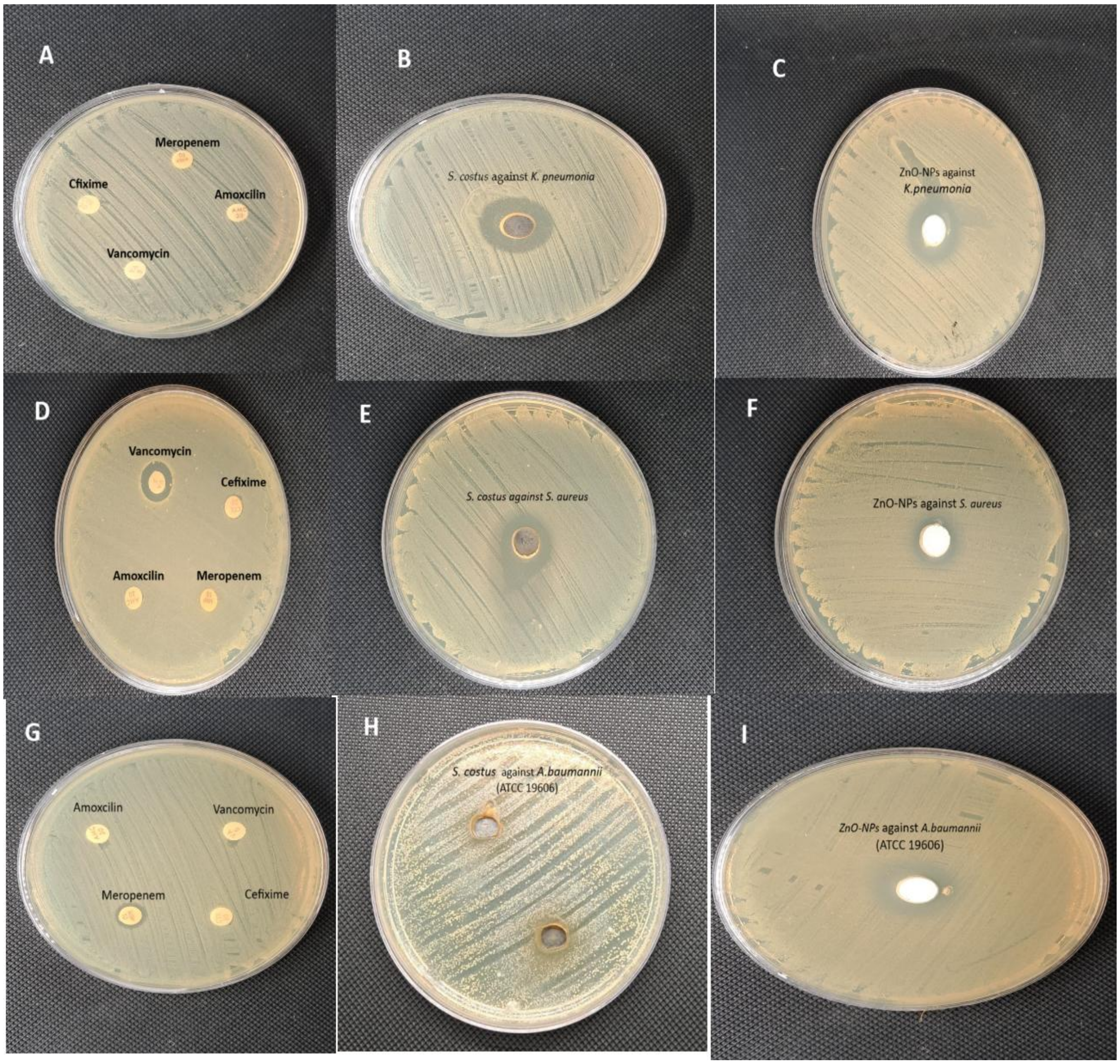

Figure 4 Anti-microbial activity of *S. costus* methanolic extract and green synthesized ZnO-NPs against *K. pneumonia* (B, C), *S. aureus* (E, F), and *A. baumannii* (H, I).

4. DISCUSSION

ZnO-NP synthesis was thoroughly investigated by many researchers who used a wide range of biological substances. The production of ZnO-NPs was evidenced by the formation of white cloudiness in the reaction mixture's solution (Vidya and Arulpandi, 2016; Ehsan and Sajjad, 2017; Mohd et al., 2020). Previous studies that utilized microorganisms and plants to synthesize ZnO-NPs revealed absorption peaks at a similar range to this study. ZnO-NPs were synthesized by Mohd et al., (2020) using bacterial cells and Lactobacillus plantarum TA4 cell-free filtrate. There were absorption peaks at 349 and 351 nm, respectively, according to the study of

the UV-vis absorption spectrum. Some investigators produced ZnO-NPs of an absorption band of 360 nm by reacting Ficus carica leaf extract with zinc sulfate as a precursor (Ehsan and Sajjad, 2017).

Our findings were corroborated by a recent study that described the environmentally friendly production of ZnO nanoparticles utilizing Cayratia pedate leaf extract (Jain et al., 2011). Previous publications provide substantial support for the current findings (Anitha et al., 2018; Alamdari et al., 2020; Vidya et al., 2013). Additionally, these results could advocate the capping of Bio-ZnO-NPs with the combined mixtures of polyphenols, proteins, and flavonoids in the *S. costus* ethanolic extract (Sathishkumar et al., 2017). In this study, methanolic extract and ZnO-NPs exhibited anti-microbial activity with a variable diameter of inhibition zones. Gram-negative strains were effectively inhibited by ZnO-NPs. This is attributed to morphological variation in the bacterial cell walls between both types of bacteria, due to the presence of peptidoglycan sheets (Devagi et al., 2020; Chatterjee et al., 2015).

Some studies contradict these findings Dayma et al., (2019), Bakhtiari-Sardari et al., (2020), and others notified a diversity of bacterial sensitivity (Składanowski et al., 2016; Wypij et al., 2018). Furthermore, the methanolic extract of *S. costus* had anti-microbial activity against all tested strains except P. aeruginosa clinical isolate. The influential anti-microbial power of *S. costus* was evidenced against numerous pathogenic bacterial species (Chang et al., 2011). We noticed that ZnO-NPs have MIC range of 3.75-7.5 mg/mL, while the MICs values of *S. costus* methanolic extract ranged from 3.75 mg/mL to 15 mg/mL. The MBC values were generally found to be twice the inhibitory concentrations.

The results of the present study are parallel to the findings of several studies wherein plant extract-mediated synthesis of ZnO-NPs offered significant MIC activity below 0.5 mg/ mL against both Gram-positive and Gram-negative bacteria (Mahendra et al., 2017). The size and the colloidal state of nanoparticles contribute to the variation in MIC values (Nateghi and Hajimirzababa, 2014). The bacteria are more susceptible to the small NPs at sizes less than 50 nm because of the facilitated diffusion (Dakal et al., 2016). The NPs interact with the bacterial outer membrane, where the electrostatic interaction of negatively charged microbe cell membranes with positively charged NPs leads to morphological alteration, degeneration, and bacterial lysis.

The lack of DNA replication ability following treatment with the metal ion may be the reason for the microorganisms' inhibitory activity. This is explained by the inactivation of other cellular and ribosomal subunit protein expression by reactive oxygen species or ROS (Yu et al., 2013; Marslin et al., 2018). Multi-drug resistance is a global health problem. Using traditional herbal extracts and their NPs in solving such problems is worthwhile. This study proved the anti-bacterial action of *S. costus* methanolic extract and NPs against MDROs. Some financial limitations precluded further testing other bacteria strains and evaluating the synthesized NPs with more advanced methods.

5. CONCLUSION

Multidrug-resistant bacteria are a disturbing maze for healthcare workers. This study provides promising alternative treatment for such a problem. The methanolic extract and NPs of *S. costus* exhibited a potent effect against G-negative and positive multidrug-resistant bacteria with variable zones of inhibitions. These findings open the way for further research on the anti-microbial effect of *S. costus* against other multidrug-resistant strains.

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Author Contributions

SE designed and supervised the study. YH, analysis of Nano studies. HN analysed the results and statistical data. All authors contributed to the writing and approved the final draft.

Ethical approval

REC-TP (Research Ethics Committee-University of Tanta (TP-RE/7/24 p-03) approved the study.

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Conflict of interest

The authors declare that there is no conflict of interests.

Data and materials availability

All data sets collected during this study are available upon reasonable request from the corresponding author.

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